

**THE ROLE OF MICROGLIA IN ADDICTION IN A
MOUSE MODEL**

by

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Abstract

Microglia, the immune cells of the brain, have traditionally been studied solely for their immunological functions. Here, we suggest a novel involvement of microglia in the development and maintenance of addiction to opioids and other drugs of abuse. In response to drugs of abuse such as morphine, microglia shift to an activated state that involves upregulated release of cytokines and chemokines, the small cell-signaling protein molecules of the immune system. Previous work in our laboratory has demonstrated that increased expression of the anti-inflammatory cytokine, IL-10, inhibits morphine-induced glial pro-inflammation within the nucleus accumbens (NAcc), and prevents morphine relapse. We therefore seek to translate these findings to a mouse model in order to further our research through transgenic manipulations. Through this project, we verify the viability of a mouse model through the elucidation of the molecular response to morphine administration in mice. We identify the optimal time point (e.g. 60 minutes) for identifying the molecular profile of morphine administration due to optimal morphine activity. Additionally, important patterns of cytokine expression observed in rat studies involving toll-like-receptor (TLR)4, CCL4, and neuronal fractalkine, are reproduced in mice. TLR4 has been implicated as a major player in addiction; therefore, we also tested the learning and memory abilities of a TLR4 knockout (KO) mouse through the well-established fear-conditioning paradigm since addiction is closely related to cognition. TLR4 KO behavior analysis shows no significant difference in fear conditioning between male TLR4 heterozygote (HET) and KO mice. Densitometry analysis also shows no difference in microglia density in these animals. Collectively, these results confirm the viability of a mouse model in continuing our research, investigating the role of microglia in addiction.

Introduction

The neural mechanisms of addiction, despite being a major socioeconomic burden and a topic of vast research, remain poorly understood. Microglia, the immune cells of the brain, have traditionally been studied for their immunological functions such as destroying invading microorganisms, scavenging for harmful debris, and acting as antigen-presenting cells that activate T-cells, in order to regain homeostasis [1]. However, recent literature has shown increasing evidence that glial cells play an important role in mediating addiction. A majority of addiction research has focused primarily on the neuronal mechanisms of addiction, but here, we present the novel involvement of microglia in the development and maintenance of addiction. Previous work in our laboratory demonstrates that microglial activation, brought on by drug use, may play a role in mediating addiction. We seek to further this research, originally done in rats, by translating this work to a mouse model in order to capitalize on the opportunity for transgenic manipulation. This work is increasingly important because the elucidation of the neuronal mechanisms of addiction may aid us in identifying liability and preventing addiction risk and relapse.

The word 'glia' is an encompassing term for the primary immunocompetent cells of the CNS that includes microglia and astrocytes [2]. Microglia are a specialized subset of macrophages in the CNS that actively scan tissue for damage, which challenges the traditional notion of quiescent microglia in the healthy brain [3]. In response to an immune challenge, microglia are activated by cytokines produced by nearby immune effector cells, such as T-cells, or by surface structures and DNA/RNA of viral, bacterial, or fungal origin such as bacterial lipopolysaccharide (LPS) among other triggers [3]. This activation may be better described as a change in phenotype rather than a general awakening because of the continual screening activity that takes place in the normal brain [3]. Glia are activated by drugs of abuse, such as morphine, and this activation leads to a multitude of effects such as altered neuronal excitability and synaptic connectivity, as well as reduced opioid-induced analgesia and increased opioid tolerance [4-6]. Glial cells generally act through triggering

intracellular cascades that result in plastic changes to the CNS, and because astrocytes and microglia can encapsulate synapses, they are in a unique position to modulate synaptic transmission [7].

Regardless of the specific mechanism through which opioids induce glial activation, it is important to acknowledge the role glial activation plays in the mediation of pain signaling and the great need to separate the mechanisms that contribute to the beneficial and detrimental actions of opioids. Understanding glial activation during opiate exposure and glial activity in relation to addiction development is significant because of the potential to create novel treatments that may increase analgesia while avoiding addiction, tolerance, or withdrawal symptoms after use. Elucidated pathways serve as potential targets for pharmaceutical intervention that may decrease the widespread socioeconomic impact of drug abuse by lessening the compulsion of addiction, reducing the risk of relapse, and identifying those individuals who are vulnerable to drug abuse.

Societal and health consequences of drug addiction

Drug addiction poses a large threat to the economic and health status of countries around the world. The insidious nature of drug use stems from the fact that addicts compulsively use drugs even when they are aware of the negative consequences, and they are vulnerable to episodes of relapse characterized by uncontrollable drug-seeking behaviors. Nearly two decades ago, the cost of drug abuse in the United States was estimated to be around 114.2 billion dollars, and this figure is projected to have increased significantly due to inflation and population growth [8]. Few substances throughout medical history have had such widespread and prolonged use as opioids, which are derived from the dried sap of the *Papaver somniferum*, or poppy plant [9]. Opiate drugs are those drugs classified by their ability to bind opioid receptors, and this family of drugs includes some of the most widely used analgesics in the U.S., such as hydrocodone (Vicodin), codeine, and morphine. Morphine, the first active alkaloid isolated from the opium poppy, is so potent an

analgesic that it is considered the 'gold standard' for opioid analgesics [9]. However, due to its potency, morphine abuse has steadily increased in recent years, becoming the second most frequently abused illicit drug amongst secondary students in the United States [10].

Another issue of concern in the healthcare sector is the development of addiction to prescribed drugs during therapy. From a pharmacological perspective, prescription drugs have the same molecular profile and mechanism of action as drugs of abuse. Several factors influence the potential risk for addiction such as dose, administration, context, and expectation. It is important to note that these risk factors apply to all forms of drug addiction and do not separate drug abusers from non-drug abusers. For some drugs, such as methylphenidate used for attention deficit disorder, the prescribed dose is typically below the level expected to produce reinforcement [11]. However, this is not always true, as seen in cases of opioid analgesics, where the dose for adequate pain control is similar to that used by abusers [12]. In some instances, non-abusers and abusers may react differently to the same dosage. For example, non-abusers may feel non-reinforcing symptoms such as discomfort while abusers experience highly reinforcing analgesia [12]. This emphasizes the fact that drug tolerance, addiction, and pleasure are highly influenced by individual differences. In fact, opioid drug abuse has a high heritability rate and a family history of opioid abuse is one of the most potent risk factors for addiction [13]. Studies comparing morphine effects on two strains of mice (C57BL/6J and DBA/2J) show that morphine administration has a differential effect based on genotype [13]. Opioid use is foreseen to continue indefinitely because of its extensive use in areas such as surgery, palliative care, and chronic pain management; therefore, it is important to continue investigating the mechanisms through which opioids act in order to define the factors that contribute to addiction and relapse liability. In light of these findings, much research over the past decade has sought to elucidate the neural pathway of addiction.

Neural substrates of addiction

The first step towards this goal is to identify which specific neural substrates are involved with drug reward in order to better understand the neural pathway that supports drug addiction. Using Fos protein expression as a marker of neuronal activity, various brain nuclei have been studied in relation to cue-induced opiate seeking [14]. Structures activated in morphine-seeking mice were the nucleus accumbens (NAcc) core, basolateral amygdala, substantia nigra pars reticulata (SNpr), and the central nucleus of the amygdala [14]. All of these brain regions are associated with the well-characterized reward pathway [14, 15]. Additionally, many studies have explored the role of the NAcc and substantia nigra pars compacta (SNpc) in addiction because of their roles in dopamine (DA) reward signaling. Activation of these specific areas supports a cortico-striatal limbic circuit that may drive morphine seeking following a period of drug abstinence [14]. However, studies must not be limited to the traditional neural structures associated with drug addiction because this limitation eliminates the possibility of finding novel brain regions that may play a role in addiction, maintenance, and extinction. Therefore, a wide range of brain regions has been tested in addition to these traditional circuits. Noteworthy substrates include the lateral hypothalamus, which may be involved in context-induced reward seeking, the NAcc shell, which is associated with drug seeking behavior driven by spatial and contextual information, and the locus coeruleus, periaqueductal gray, and lateral habenula which are related to reward expectancy and incentive value [14]. These preliminary studies have shown us that addiction pathways may not be as localized as we once thought and that continued research must be done in order to identify critical target areas.

It has been suggested that neural adaptations in the ventral striatum and ventral midbrain following chronic opioid administration contribute to the development and maintenance of opiate addiction [16]. Drugs have effects over time to induce molecular changes in addiction pathways through modifying synaptic connections. Therefore, researchers have investigated the upregulation

and downregulation of genes in the ventral striatum and ventral midbrain, such as the ventral tegmentum area (VTA) and SNpc that are associated with addiction, in order to assess the genotype-dependent regional effects of morphine [16]. Gene expression and bioinformatics analysis of the affected genes showed that these genes were associated with neuroplasticity, axonal guidance, and transcription of micro-RNAs. Of interest from the results was spinophilin, which is a dendritic spine-enriched scaffold S protein that mediates synaptic transmission and DA mediated plasticity in the striatum. Also, miRNA genes H19 and Mirg were identified as potential targets of drug-related adaptation because miRNAs influence dendritic architecture in response to activity-dependent events [16]. Taken together, these results suggest that plasticity processes may be involved with drug self-administration behavior. Interestingly, passive administration behavior, produced through a yoked control, elicits lower expression of these genes suggesting that self-administration and passive administration are distinct processes [16].

Addiction initiation and maintenance

Following the identification of potential neural substrates for addiction, it is necessary to determine the process through which addiction is initiated and maintained. For years, DA has been targeted as a crucial player in addiction because of its well-known modulation of reward signaling in the NAcc. Acute administration of all major addictive drugs causes increased DA transmission to the NAcc [17] and is important for the perception of reward and initiation of addiction. Studies using NR1-DAT-Cre mice have shown that NMDARs in dopaminergic cells are important for CPP reinstatement and drug self-administration and may be involved in recall of previously learned behaviors in response to salient stimuli [17]. These dopaminergic projections trigger a behavioral response to motivational events and show that DA signaling is important for learned behavior [18]. Once the association is learned, relapse is thought to arise from plastic changes that alter excitatory

transmission at the dendrites of DA neurons in the VTA or SNpc, which cause DA neurons to fire onto the NAcc [18].

The development of drug addiction begins with initial, acute exposures to the drug and the onset of acute drug symptoms. DA receptors in the NAcc are triggered by dopamine release from the VTA, and these receptors activate cAMP-dependent protein kinase A (PKA). This leads to PKA-induced phosphorylation of CREB, which induces immediate early gene (IEG) products such as *c-Fos*. IEGs promote plastic changes at the synapse that increase the perceived reward and, perhaps, contribute to the transition from recreational to addictive drug use [18]. Research using *drd2-eGFP* or *drd1a-eGFP* bacterial artificial chromosome (BAC) transgenic mice has revealed that there are two distinct neuronal populations in the NAcc. The NAcc is predominantly composed of medium-sized spiny neurons (MSNs) and these MSNs can be divided into two subsets; one subset expresses dopaminergic D1-type receptors (D1Rs) and the other subset expresses dopaminergic D2-type receptors (D2Rs). Chronic injection of morphine causes *c-Fos* protein expression predominantly in D2Rs [19, 20], while a single morphine injection causes *c-Fos* protein expression predominantly in D1Rs. Separation of these NAcc specific neuronal populations contributes to our understanding of the neuronal basis of addiction and other associated disorders.

Despite the strong focus on DA in modulating addiction, DA is only responsible for the initiation of addiction, and studies have shown that glutamatergic projections from the PFC to the NAcc are equally, if not more, important for maintenance [18]. These glutamatergic projections to the NAcc core are responsible for driving the drug-seeking, compulsive behavior that characterizes addiction [18]. This behavior is thought to stem from three simultaneous synaptic changes. First, alterations in G-protein signaling increase the excitability of glutamatergic neurons projecting to the NAcc. Second, the dysregulation of metabotropic glutamate inhibitory autoreceptors increases presynaptic glutamate release. Lastly, the dysregulation of postsynaptic density proteins alters the postsynaptic responsiveness to this increased glutamate release from the presynaptic terminal

[18]. While blocking the dopaminergic or glutamatergic projections to the NAcc prevents the initiation and reinstatement of addictive behavior, respectively, these results show that pharmacologically targeting the PFC glutamatergic projections to the NAcc may ameliorate the compulsive drug-seeking behavior that contributes to the pathology of addiction. Continual administration of drugs of abuse leads to tolerance, which is characterized by the need for increased drug administration in order to achieve the same amount of analgesia attained at the beginning of drug use. Tolerance is a distinctive trait of opioid drug addiction, and morphine tolerance and addiction are believed to be mediated by complex changes in neural plasticity [7]. Elucidating the neural substrates of addiction is important for understanding the cellular mechanisms of tolerance and addiction, and though NMDA receptors have been a primary focus of this research, recent literature has shown increasing evidence that glial cells may play an important role in mediating addiction.

Glial pathways

Though several lines of evidence suggest that glial activation may play a role in addiction, the specific mechanism through which this process takes place is still under investigation. It is proposed that opioids do not cause glial activation through classical opioid receptors, but instead induce glial activation through a class of pattern recognition receptors called toll-like receptors (TLRs), specifically TLR4 [4]. However, opposing evidence suggests that microglial activation is caused by a mechanism independent of the TLR4 pathway in the development of morphine tolerance [21]. The reason that the TLR4 pathway has been identified as a possible mechanism for opioid action is due to the accumulating evidence that selective, acute antagonism of TLR4 results in reversal of neuropathic pain and potentiation of opioid analgesia [4]. One novel drug, plus-(+) naloxone, has been widely used to block TLR4 activation-induced gene expression of both proinflammatory cytokines and a microglia marker CD11b [4, 22, 23]. Assuming that opioids work

through the TLR4 pathway, extensive studies have been done investigating the effects of acute TLR4 antagonism, *TLR4* genetic knockout, and blockade of TLR4 downstream signaling (e.g., MyD88) on morphine withdrawal and analgesia [22]. It has been shown that disrupting the TLR4 pathway leads to reduction of withdrawal symptoms [24] and increased analgesia [22, 24]. Other opioid manipulations have included studies using several non-specific glial activation inhibitors such as ibudilast, minocycline, and propentofylline [4, 21, 22, 24]. These drugs were used as pharmacological interventions and studied for their ability to inhibit glial activation or proinflammatory cytokine actions, which ultimately improved the clinical efficacy of the tested opioids [22]. One suggested hypothesis for the molecular mechanism of microglial activation by morphine is the binding of myeloid differentiation protein 2 (MD-2), an accessory protein of TLR4, to morphine. Morphine binding to MD-2 causes TLR4/MD-2 oligomerization and subsequent TLR4 cascade signaling [5].

In light of this hypothesis, there are opposing studies that reject the concept of TLR4 involvement in glial activation. In these studies, it is shown that glial activation mediates opioid tolerance because minocycline, a selective glial inhibitor, attenuates the development of tolerance in mice. Mutation or deletion of the *TLR4* gene, however, does not affect the development of tolerance, which suggests that tolerance is mediated by microglial activation independent of TLR4 [21]. In addition, CD11b mRNA is elevated in both *TLR4* knockouts and WTs in response to morphine [21]. Previous studies supporting TLR4 activation of glia have not focused specifically on tolerance [4, 22, 23], but tolerance is directly related to analgesia potential, which is enhanced through TLR4 antagonism. The analgesic potency of morphine is also threefold higher in *TLR4*-knockout mice compared to WTs. Therefore, tolerance may be discernible if mice are given lower doses of opioids. Current studies opposing TLR4-mediated glial activation have only investigated results based on WT and *TLR4*-knockout mice given equivalent doses, but it is speculated that similar tolerance is induced [21]. Given these data, it is still controversial whether opioids cause

glial activation through the TLR4 cascade or another closely related nonclassical opioid receptor pathway. Other targets of interest include TLR2 and P2X4 and P2X7 receptors [21].

The basic hypothesis for the mechanism through which glial activation affects morphine tolerance is separated into two steps. First, it is proposed that morphine acts directly on glial cells and alters their metabolism, morphology, and function [7, 25]. This activation induces increased activity of glial cells, which release a variety of substances that have been associated with morphine tolerance, such as nitric oxide, an important cellular signaling molecule, and cyclooxygenase products, along with multiple neurotransmitters that contribute to neuronal plasticity [7, 25]. Glial cells are also responsible for the uptake of amino acid neurotransmitters, such as GABA and glutamate, and are therefore intimately associated with synaptic transmission regulation [7]. Activated glia that contribute to morphine-induced reward are generally found in the NAcc, but the role of microglia in this region remains unclear [26]. One recent study shows that chronic morphine treatment induces activation of p38 in NAcc microglia [26]. p38 is a mitogen-activated protein kinase (MAPK) that may be associated with degenerative disease of the CNS and may mediate morphine-induced responses like tolerance and analgesia [26]. Conditioned place preference (CPP) is a paradigm used to demonstrate the acquisition of addiction. Mice are conditioned to associate a certain context with a drug and another context with the vehicle in which the drug was administered. After the association has been acquired, the strength of the association between context and drug is assessed through measuring the time spent in both the drug and vehicle associated contexts. This paradigm can also be used to study the tendency for relapse if mice are exposed to the drug context without administration of drug (extinction phase) and then later reintroduced to a priming (low or threshold) dose of the drug. Studies show that p38 inhibitor (administered before morphine treatment) prevents acquisition of CPP. In addition, minocycline, a microglia inhibitor, was also able to prevent CPP acquisition. Interestingly, p38 inhibitor and minocycline were not able to prevent expression of CPP after it was established; however, chronic

injection of the p38 inhibitor was able to interrupt CPP maintenance. The need for *chronic* injection of an inhibitor in order to attenuate CPP maintenance suggests that these effects may take place through the long-term release of cytokines and neurotrophic factors from activated microglia. Cytokines and neurotrophic factors contribute to long-term adaptations in the neural circuit [27]; therefore, it is plausible that these molecules may have an effect on drug addiction substrates. These results suggest that p38 signaling in NAcc microglia may be critical for acquisition and maintenance, but not expression of morphine CPP [26]. However, p38 is not exclusive to microglia, so these data must be interpreted with caution.

Early-life experiences

In addition to elucidating the neural and cellular mechanisms of addiction, it is important to identify the environmental factors that play a role in initiating and mediating addiction. Several factors such as genetics, physiology, and environment, specifically early-life environment, have been shown to significantly affect an individual's risk of addiction. Early life stressors in rodents increase drug self-administration [28], alcohol intake [29], and alter CPP [30], suggesting that early-life experiences may program the brain and prompt behaviors that ultimately impact neural function in adulthood and contribute to addiction liability. Recent literature has shown that naturally occurring variations in maternal care mediate significant variations in offspring cognitive development [31]. Handling paradigms are used to manipulate the quality and quantity of maternal care, which includes behaviors such as licking, grooming, and arched back nursing. Handling paradigms intended to increase maternal care behaviors separate mothers from their pups for a period of at least 15 minutes before returning them to the same cage. This brief separation induces an innate increase in maternal behaviors as compared to that of mothers who remained undisturbed [31]. Neonatal handling paradigms have shown that early life experiences change the responsiveness of a) mesocorticolimbic DA neurons to stress and psychostimulants [32], b) the

microglial activation in response to an immune challenge [31], and c) the rewarding effect of morphine and tendency for morphine relapse [2]. Recently in our laboratory, handling experiments in rats have shown that maternal care attenuates morphine-induced glial activation, increases basal levels of anti-inflammatory cytokine interleukin (IL)-10 within microglia in the NAcc, and prevents morphine relapse in adulthood [2]. The increase in basal IL-10 occurs because of decreased methylation of the *IL-10* gene specifically within microglia. These neonatal handling effects can be replicated pharmacologically in adults with administration of ibudilast, a glial modulator. Ibudilast increases IL-10 expression, inhibits morphine-induced glial activation within the NAcc, and prevents morphine relapse. These novel findings suggest that IL-10 may modulate the negative effects of microglial activation in response to morphine and that increases of IL-10 in the NAcc may decrease the tendency for relapse, especially in vulnerable individuals [2]. Based on this previous work, we hope to establish a causal relationship for microglial IL-10 in drug resilience.

General Aims

In order to translate our previous research to a mouse model, it is necessary to investigate the molecular expression profile of mice in response to morphine administration. With these exploratory experiments, I seek to examine the following questions: 1) in mice, is there an effect of time, dose, or brain region on glial activation in response to morphine administration? and, 2) Given the evidence suggesting a possible TLR4 mechanism of glial activation, is there a TLR4 gene dose response in mice with respect to learning? The latter question stems from the fact that learning is an important measure of cognition, and cognition is closely related to all addiction processes.

Materials and Methods

Experiment 1: Morphine Exposure in Mice

Forty-eight adult male C57 BL/6 mice were obtained from Charles River Laboratories (Raleigh, NC) and housed in ventilated polypropylene cages with *ad libitum* access to food and water. The colony was maintained at 22°C on a 12:12 h light- dark cycle (lights on at 7:00 A.M. Eastern Standard Time).

Injections and Perfusions

The 48 mice were divided into 6 groups based on 2 different morphine doses and 3 sacrifice time points. This resulted in 7 animals per dose/time point combination group plus one mouse per time point group designated as a saline control. Animals were sacrificed at either 5, 40, or 60 minutes following morphine or saline administration. Morphine-treated animals were given 3 mg/kg or 10 mg/kg subcutaneous (s.c.) morphine injections. Controls were given s.c. saline injections. At the designated time point, mice were anesthetized with 0.2 mL of a ketamine-xylazine cocktail and sacrificed by transcardial perfusion with 0.9% saline. Brains were then extracted and cut in half. One hemisphere was placed in a 4% paraformaldehyde solution for histology. Prefrontal cortex, nucleus accumbens (NAcc), and hippocampus (HP) samples were extracted from the remaining hemisphere and immediately flash frozen in isopentane for later RNA extraction and PCR analysis.

Tissue RNA extraction

The frozen samples from the nucleus accumbens and the hippocampus were homogenized in TRIzol (Life Technologies, Grand Island, NY) and RNA was precipitated using chloroform:glucagon:isopropyl alcohol extraction. Following RNA isolation, the samples were treated with DNase, and complementary DNA (cDNA) was synthesized from 100 ng of isolated RNA using the Qiagen QuantiTect Reverse Transcription Kit (Valencia, CA, USA) or from 500 ng of isolated RNA using the RT² First Strand Kit (SABiosciences/Qiagen, Frederick, MD, USA).

Quantitative Real-time PCR and Analysis

Gene expression was measured using quantitative real-time PCR with primers designed to measure mouse cytokines, chemokines and receptors using the RT² SYBR[®] Green qPCR Master Mix (Cat. No. 330500, Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Seven mice per treatment group plus saline controls were analyzed according to this method.

qRT-PCR analysis. Threshold amplification cycle number (Ct) was determined for each reaction within the linear phase of the amplification plot and relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method [33, 34]. Relative gene expression across groups was compared using either a) a three-way ANOVA with brain region (HP or NAcc), dose (saline, 3 mg/kg, or 10 mg/kg), and time (5 min., 40 min., 60 min.) as factors or b) a one-way ANOVA with time point as a factor, comparing only NAcc tissue from mice that received either saline or 3 mg/kg of morphine.

Primer specifications.

Real-time quantitative PCR primers were obtained from SABiosciences/Qiagen from Cat No. PAMM-022ZA or designed by our laboratory and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sequences: GAPDH: Fwd – GGTCACCAGGGCTGCCATTT, Rev – TGGGCTTCCCGTTGATGACA; IL-10 (IL-10): Fwd – GGACAACATACTGCTAACCGAC, Rev – TGCTCCTTGATTTCTGGGC; Cd11b: Fwd – CTATTTGTTTCGGCTCCAAC, Rev – GCATCAAAGAGAACAAGG; CCL4: Fwd – GATTACTATGAGACCAGCAG, Rev – CTCCAGCGGCAGGGCAGG; CCL17 Fwd – TGAGGCATTTGGAGACGCCA, Rev – GCACTACAGGCGAACACA; CCL25 Fwd – ATAATCCCCCAACCTGTGCTC, Rev – TGAGTGTGTGAGAGTGCCTA.

Experiment 2: Learning and Memory in TLR4 HET and KO Mice

Knockout (KO) mice are genetically engineered to have a missing or disrupted copy of a specific gene. Heterozygous (HET) mice have one copy of the gene that is missing or disrupted, while the

other copy of the gene is intact. These mice were bred from a TLR4^{+/-} HET female and TLR4^{-/-} KO male; therefore, we could compare TLR4 HET and KO littermates to see if there was a gene dose effect on learning or memory. Ten adult male and 10 adult female TLR4 HET and TLR4 KO mice bred on a C57 BL/6 background were obtained from the laboratory of Dr. Richard Auten at Duke University and housed in ventilated polypropylene cages with *ad libitum* access to food and water. The colony was maintained at 22°C on a 12:12 h light- dark cycle (lights on at 7:00 A.M. Eastern Standard Time).

Fear conditioning

Addiction relies heavily on cognitive associations made between drugs and physiological or environmental cues. This close relationship is a result of the strong role that cognitive association plays in forming patterns, habits, and general addictive behaviors. Therefore, intact learning is a necessary measure used to verify transgenic animal model viability. Fear conditioning paradigms are routinely used to measure the cognitive ability of mice to form associations between conditioned and unconditioned stimuli. Therefore, we compared TLR4 knockout mice's performance in a fear-conditioning paradigm with that of wild type mice in order to determine if cognition has been compromised as a result of transgenic breeding.

All subjects received three pairings of an auditory cue (tone; conditioned stimulus (CS)) and 2-s 0.8mA shock (unconditioned stimulus (US)), with 2 minutes of exploration before the first tone-shock pairing and 90 s of exploration between each of the subsequent pairings. Mice were carried from their cages to and from the testing chamber in identical black (males) or red (females) buckets to establish a specific routine and environment for contextual fear testing.

Memory testing and fear conditioning scoring

Forty-eight hours following conditioning, mice were given a contextual fear conditioning memory test, which consisted of 6 minutes in the original testing chamber. Contextual fear conditioning relies on the hippocampus and involves forming an association between the testing chamber

environment and the aversive footshock. The same containers used to carry the mice during conditioning were used during memory testing. Three hours following the contextual test, mice were given an auditory test, which consisted of a 3-minute pre-CS period and a 3-minute CS period in a novel testing chamber. Auditory-cued fear conditioning relies on the amygdala and involves forming an association between the tone cue (CS) and the aversive footshock (US). During both the contextual and auditory tests, mice were scored for freezing behavior by two independent raters. Every 10 s, each mouse was judged as either freezing or active. Freezing is defined as the absence of visible movement, excluding respiration. Mice were scored for freezing behavior during the entirety of the testing period, which began immediately after the mouse was placed in the testing chamber.

Sacrifice and Immunohistochemistry for Iba1

Forty-eight hours following memory testing, animals were deeply anaesthetized with 0.2 mL of a ketamine-xylazine cocktail and sacrificed by transcardial perfusion of 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB). Brains were extracted and stored in a 4% paraformaldehyde solution for 2 days. Then brains were then transferred to a 30% sucrose and 0.1% sodium azide solution to cryoprotect them before slicing. Brains were exhaustively sliced at 40 μ m on a cryostat and stored as free-floating sections in 0.1% sodium azide. Free-floating sections were initially rinsed for 3 \times 5 min in 0.01 M phosphate-buffered saline (PBS) and also rinsed before each subsequent step, excluding the step between the blocking and primary antibody steps. Next, sections were washed in 50% methanol for 30 min. Sections were then quenched in 0.6% hydrogen peroxide for 30 min and then blocked for another 30 min in 5% normal goat serum and 0.3% Triton-X to block and permeabilize, respectively, in PBS (blocking buffer). Following this blocking step, sections were incubated overnight at room temperature in Iba1 antibody (1:10,000, rabbit polyclonal, Wako Pure Chemical Industries, Ltd.) in blocking buffer. The following day, sections were incubated for 2 h at room temperature in a solution of biotinylated anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA, USA) in blocking buffer. The Avidin-Biotin

Complex (ABC) method was used to bind a complex of streptavidin–biotin peroxidase to the secondary antibody (1 h incubation), which was then developed with diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) for 20-45 min to produce a colorimetric stain. Sections were mounted on gel-coated slides, dehydrated and coverslipped with Permount.

Densitometry for TLR4 HET and KO brains

Quantification of Iba1-positive cells was performed using ScionImage densitometry. Five sections per mouse were analyzed for the dentate gyrus, CA1, and CA3 sub-regions of the hippocampus. Digitized images of each region of interest (ROI) were taken at 10X using a Nikon Eclipse 80i microscope and digital camera on a Dell PC running PictureFrame software. Each region was traced using ScionImage after converting the image to grey scale. Signal pixels of an ROI were defined as having a grey value of 3 SDs above the mean grey value of a cell-poor area close to or within the ROI. The number of pixels and the average grey values above the set background were then computed for each ROI and multiplied by the area of the traced ROI, resulting in an integrated area density measurement. All values for each of the 5 sections per rat were averaged to obtain a single integrated density value per sub-region of interest for each rat. A two-way ANOVA was used to analyze the effects of genotype and sex on the individual values obtained for each rat.

Statistics

Data for morphine exposure in mice was analyzed using a 3-way ANOVA comparing time point, dose, and region. Based on the present data, we performed several exploratory *t*-tests to investigate differences between time point values. IL-10 values are typically hard to obtain because of the nature of expression; therefore, the limited data available was used to drive the statistics for IL-10. Ongoing analyses for the Iba-1/IL-10 double label tissue is ongoing and should show us if IL-10 protein is upregulated or specifically distributed in either the HP or NAcc. Data for TLR4 HET and KO mice was analyzed using a 2-way ANOVA comparing sex and genotype.

Results

Experiment 1: Morphine Exposure in Mice

To assess the response to morphine with respect to time and dose, we measured the gene expression of several important inflammatory markers and microglial cytokines and chemokines using real-time PCR. We find that longer time points may be important for morphine administration and result in an increase in specific cytokine production. Our results show a trending difference in CCL4 with respect to dose by region ($F_{(2, 93)} = 2.64, p=0.078$), with a greater response in the NAcc compared to HP at lower doses and a greater response in the HP compared to NAcc at higher doses (Fig. 1). Also, for CCL4, there was a trending difference between the 5 and 60 minute time points in the NAcc ($t(25) = -1.83; p=0.0792$, two-tailed), but not in the HP ($t(25) = -1.06; p= 0.299$, two-tailed). CCL17 (Fig. 2) gene expression was significantly increased in the HP compared to the NAcc ($F_{(1,93)} = 38.65; p<0.001$), while CCL25 (Fig. 3) expression was significantly increased in the NAcc compared to the HP ($F_{(1,93)} = 7.20; p= 0.009$). Additionally, IL-10 differed in expression between 5 and 40 minutes time points in HP (Fig. 4), but not in the NAcc ($t(22) = -2.47; p= 0.022$) (Fig. 5).

We also utilized a PCR array to assess expression of 85 immune genes in NAcc tissue samples. Our results demonstrated genes that are possible candidates for further, more detailed analyses. The following genes showed significant changes in expression with respect to experimental time points post morphine injection (Fig. 6): CCL4: $F(3,8) = 6.122, p=0.018$; CCL8: $F(3,8) = 4.899, p=0.032$; CCR6: $F(3,8) = 4.945, p=0.031$; CMKLR1: $F(3,8) = 9.271, p=0.006$; CXCR7: $F(3,8) = 5.04, p=0.03$; FPR1: $F(3,8) = 8.767, p=0.007$; GPR17: $F(3,8) = 13.681, p=0.002$; Itgb2: $F(3,8) = 4.745, p=0.035$; MAKP14: $F(3,8) = 4.233, p=0.046$; CX3CL1: $F(3,8) = 7.13, p=0.012$; CX3CR1: $F(3,8) = 6.006, p=0.019$ (Fig. 7).

Experiment 2: Learning in TLR4 HET and KO Mice

TLR4 HET and KO mice were fear conditioned and given contextual and auditory-cued memory tests to assess learning. Our results show that contextual fear conditioning differs by sex, with females freezing significantly more than males ($F_{(1,19)} = 7.541$; $p = 0.014$) (Fig. 8). In contrast, results of the CS conditioning (auditory-cued) show a difference by genotype with KOs freezing significantly less than their HET counterparts ($F_{(1,19)} = 6.06$; $p = 0.026$) (Fig. 9). This difference in genotype is primarily driven by female HETs freezing more than their KO counterparts, while male HETs and KOs demonstrated lower freezing overall.

Discussion

Increasing lines of evidence suggest that microglia play a significant role in pathological pain and addiction. Here, we investigate the effects of morphine administration on microglia and neuroimmune signaling in mice in order to create a molecular profile for this animal model for its future use in addiction studies. The results of our exploratory experiments suggest that, in mice, morphine may have the greatest effect on microglial activation at longer time points (e.g., 60 minutes) than originally demonstrated in previous work with morphine administration in rats [2]. These experiments also show differential cytokine/chemokine expression in the NAcc as compared to the HP in response to morphine administration, indicating that microglia may behave in a heterogeneous manner depending on their location in the brain. Our PCR array also confirms some of the results achieved through single gene real-time PCR. CCL4 showed a significant expression difference in both the single gene analysis and PCR array, with increased gene expression visualized at 60 minutes post morphine injection when compared to shorter time points. Other significant differences were seen in CCL7, CCR6, CMKLR1, CXCR7, FPR1, GPR17, Itgb2, MAKP14, CX3CL1, and CX3CR1. CX3CL1 and CX3CR1 are commonly known as fractalkine, a neuronal chemokine, and fractalkine receptor, expressed on microglia, respectively. Increases in neuronal fractalkine

expression in response to morphine administration, mimics the response seen in rats. [2] Also, several trends were seen in expression levels of CCL3, CCR3, CXCL13, CXCR3, CXCR4, Itgam, Ppbp, and TLR4 with respect to different experimental time points in the NAcc. Overall, the subset of significant chemokine expression changes in mice differed from that seen in rats, excluding neuronal fractalkine [2], suggesting that chemokine response profiles may be species specific, in some cases.

In light of our laboratory's previous findings, which suggest a novel role of IL-10 in blocking microglial activation and relapse in rats, we have continued to investigate the expression of IL-10 in mice. Though it is not clear if IL-10 gene expression differs significantly in the NAcc and HP due to the difficult nature of measuring IL-10, we are currently investigating IL-10 protein expression through an Iba-1/IL-10 immunofluorescent IHC analysis. If we find that IL-10 protein expression or distribution is significantly different in response to morphine administration, this may suggest that its altered expression can affect drug addiction liability. Additionally, in light of our laboratory's findings that correlate neonatal handling with an increase in IL-10, confirmation of IL-10 involvement in drug addiction in mice would suggest that early life experience might impact addiction resilience in adulthood.

Several authors have implicated the TLR4 pathway in association with microglial activation and addiction; therefore, we investigated whether there is a gene dose response of TLR4 on learning. As previously mentioned, learning is an important marker for ensuring proper cognitive development, and cognition is closely related to all addiction processes. We use the well-established fear-conditioning paradigm as a measure of cognitive viability for our mouse model. Our preliminary study shows that female HETs and KOs freeze considerably more than their male counterparts. In addition, HETs freeze significantly more to auditory cues than KOs; however, increased freezing in female HETs is the primary driving force for this main effect. In addition, densitometry analysis of TLR4 HET and KO brains showed that there is no significant difference in

microglia density or distribution in the hippocampus with respect to genotype. The PCR array results show a trending increase of TLR4 60 minutes post morphine injection. This result suggests a dose response of TLR4, which mimics the overall increases seen in other cytokine/chemokine expression at longer time points. Collectively, these results are important because they suggest that the cellular profile of TLR4 HET and KO mice are similar; however, TLR4 KO may affect learning and thus may not be a suitable model for future investigation of TLR4 involvement in morphine addiction acquisition, development, and maintenance. A microglia-specific TLR4 KO may be a potential future model for TLR4 investigation. Still, caution is needed with regard to behavioral models of addiction that involve learning (e.g., CPP).

There are still several questions we must address in continuing this microglial addiction research in mice. First, we must verify the trends that we have seen in cytokine/chemokine expression and further investigate the relationships between dosage, time point, and gene expression. Secondly, we must determine if IL-10 protein expression in the mouse brain mimics the same pattern of IL-10 expression previously measured in rats [2]. Answering these two questions will allow us to more confidently translate our previous results to a mouse model, and further our addiction research through the use of transgenic mice.

Microglia and other glial cells have become a novel substrate for investigation in the field of addiction research, yet many questions still remained unanswered. Through our experiments, we hope to elucidate the time course of microglial activity, the species specificity of microglia involvement in addiction, and the overall necessity of microglial activity in addiction, or lack thereof. The importance of these experiments, collectively, stems from the urgent need to understand the molecular profile and consequences of drug use in order to predict addiction and relapse. Drug addiction is incredibly debilitating in terms of socioeconomic and health status across the world. Understanding the molecular consequences of drug use may ameliorate the difficulty of

drug addiction treatment and allow us to more effectively treat addicted patients and prevent addiction and relapse in vulnerable individuals.

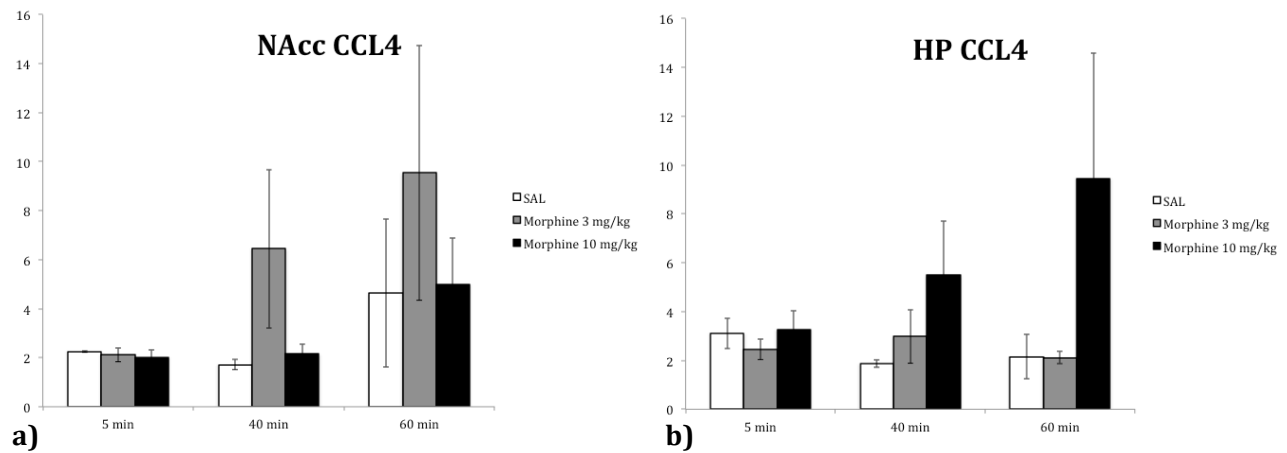


Figure 1. CCL4 expression shows a trending difference between the 5 and 60 minute post morphine injection time points in the nucleus accumbens ($t(25) = -1.83$; $p = 0.0792$, two-tailed), but not in the HP ($t(25) = -1.06$; $p = 0.299$, two-tailed). CCL4 gene expression in the nucleus accumbens and hippocampus is shown here with respect to time and morphine dose.

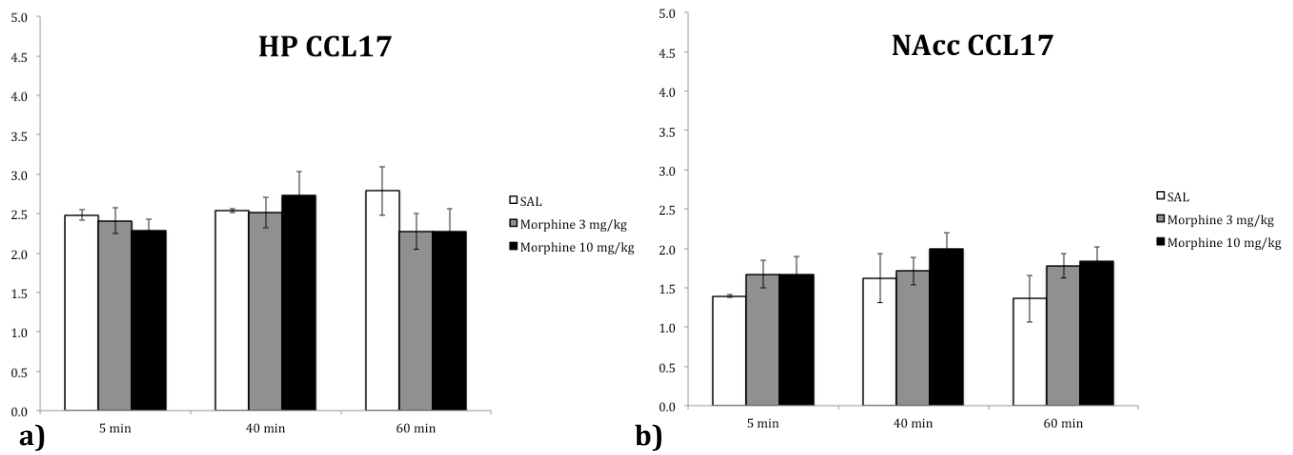


Figure 2. CCL17 gene expression was significantly increased in the HP (a) compared to the NAcc (b) ($F_{(1,93)} = 38.65$; $p < 0.001$). CCL17 gene expression in the hippocampus and nucleus accumbens is shown here with respect to time and morphine dose.

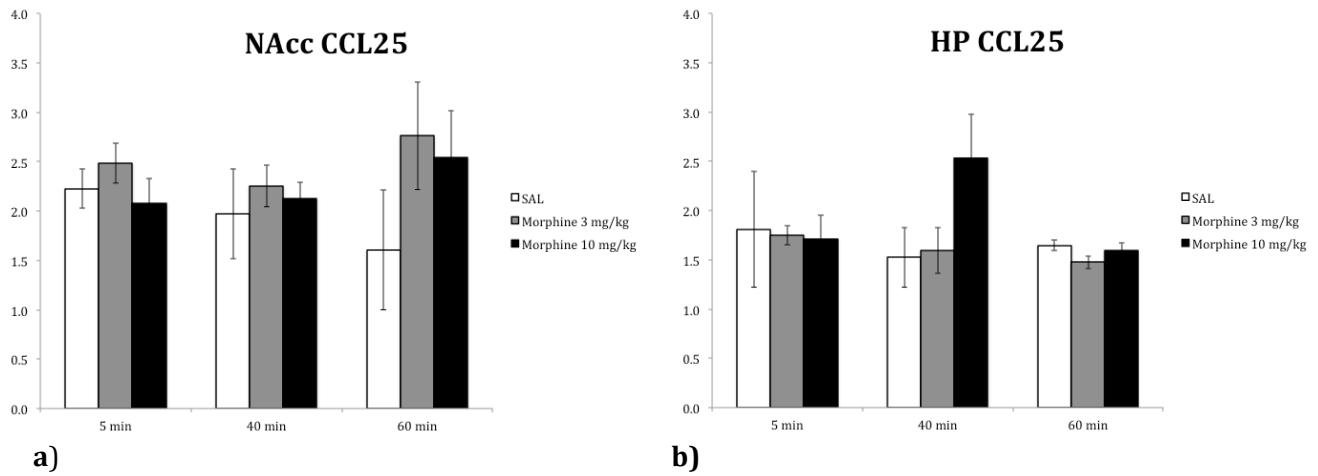


Figure 3. CCL25 gene expression was significantly increased in the NAcc (a) compared to the HP (b) ($F_{(1,93)} = 7.20$; $p = 0.009$). CCL25 gene expression in the nucleus accumbens and hippocampus is shown here with respect to time and morphine dose.

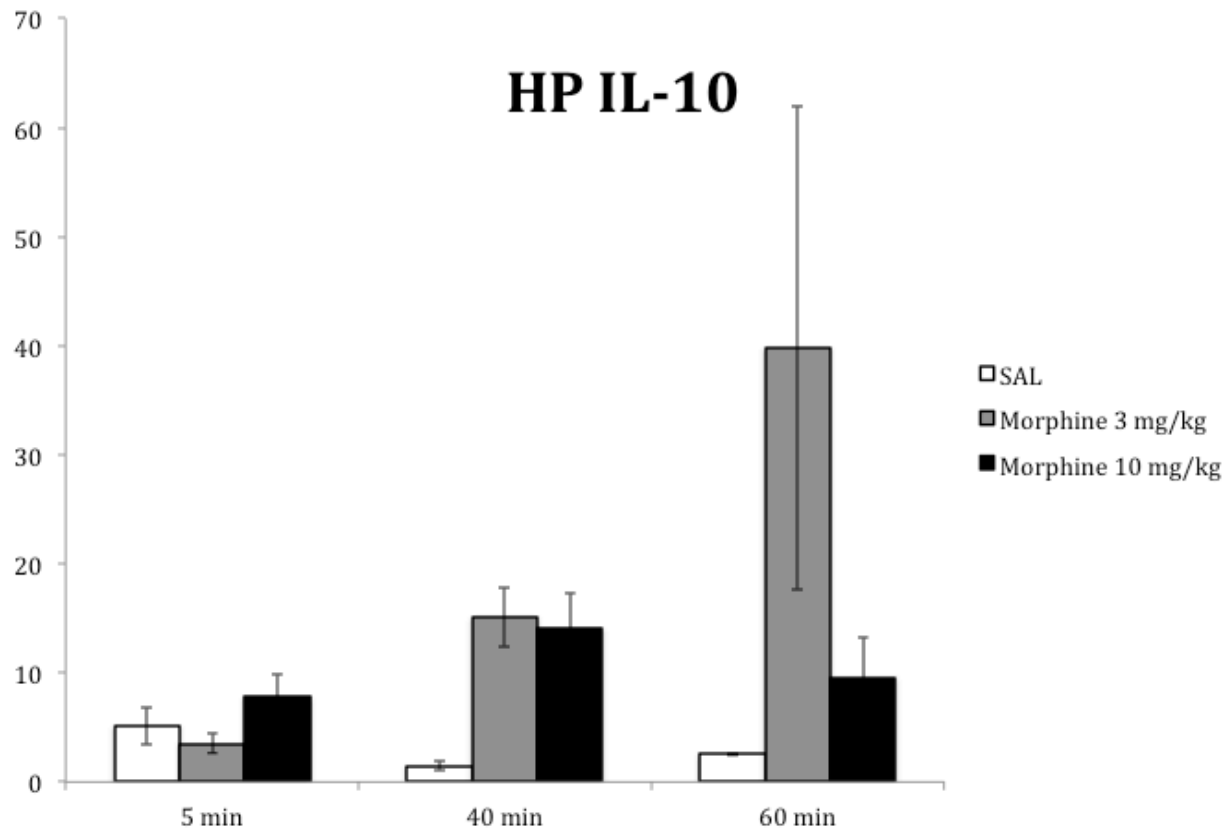


Figure 4. IL-10 gene expression differed between 5 and 40 minutes post injection time points in HP, but not in the NAcc ($t(22) = -2.47$; $p = 0.022$). IL-10 gene expression in the hippocampus is shown here with respect to time and morphine dose.

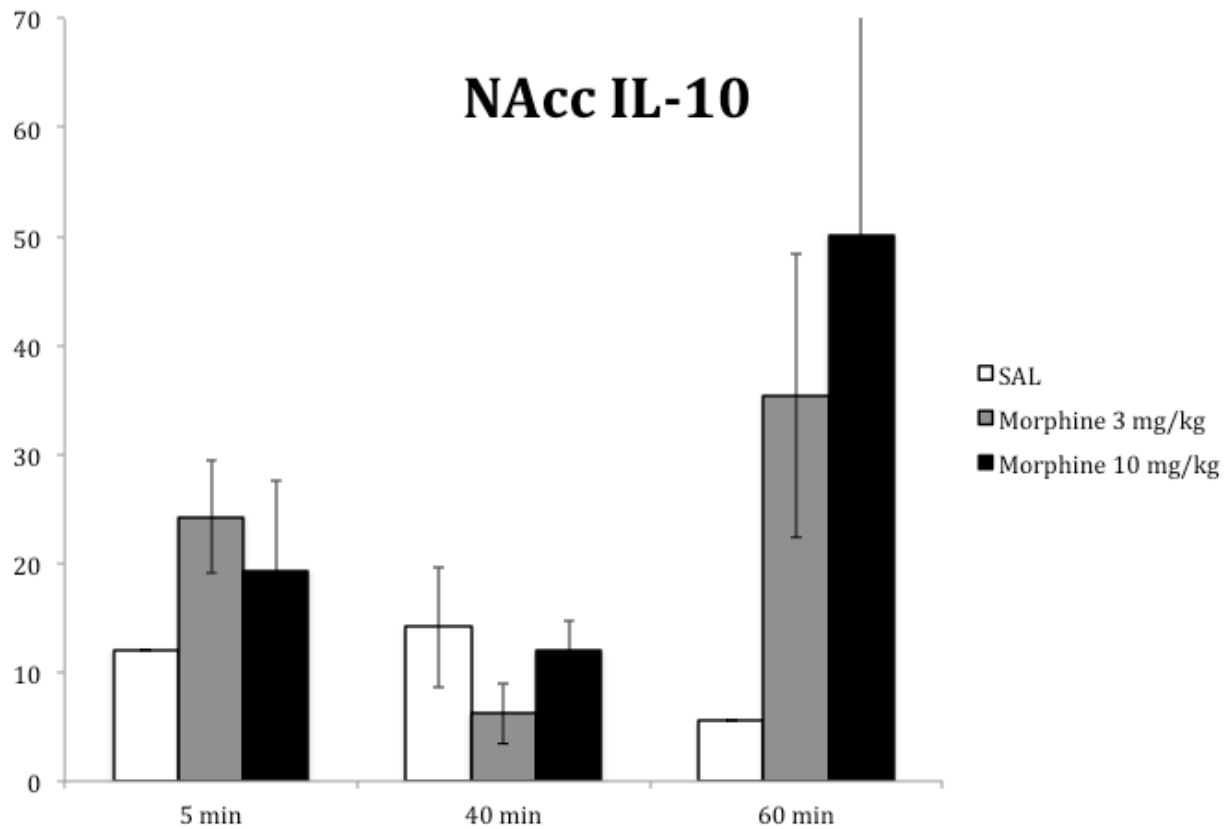


Figure 5. IL-10 gene expression differed between 5 and 40 minutes post injection time points in HP, but not in the NAcc ($t(22) = -2.47$; $p = 0.022$). IL-10 gene expression in the nucleus accumbens is shown here with respect to time and morphine dose.

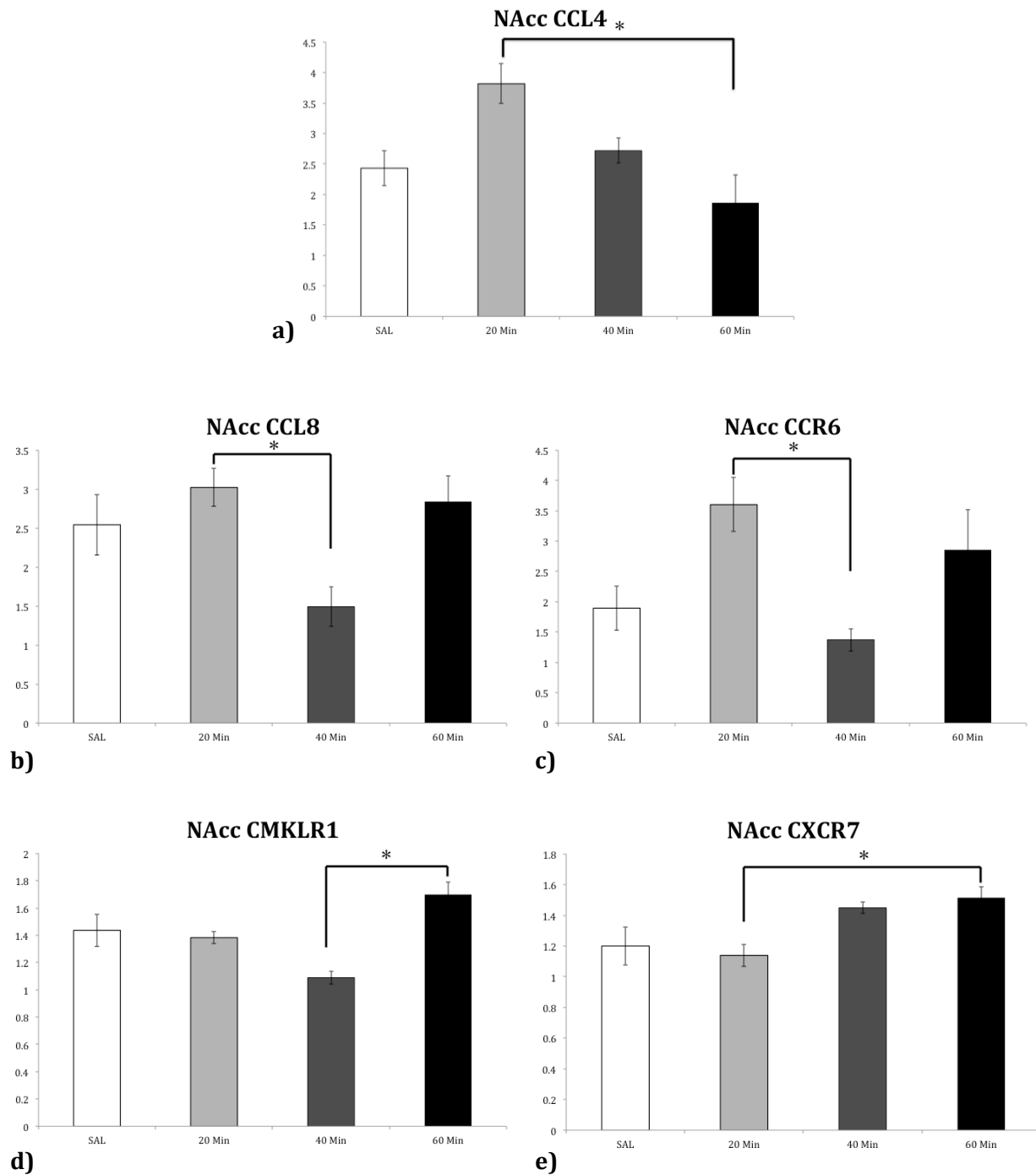


Figure 6. a) CCL4 expression in the NAcc shows significant difference between the 20 min and 60 min post injection time points. b) CCL8 expression in the NAcc shows significant difference between the 20 min and 40 min post injection time points. c) CCR6 expression in the NAcc shows significant difference between the 20 min and 40 min post injection time points. d) CMKLR1 expression in the NAcc shows significant difference between the 40 min and 60 min post injection time points. e) CXCR7 expression in the NAcc shows significant difference between the 20 min and 60 min post injection time points. Chemokine expression in the NAcc.

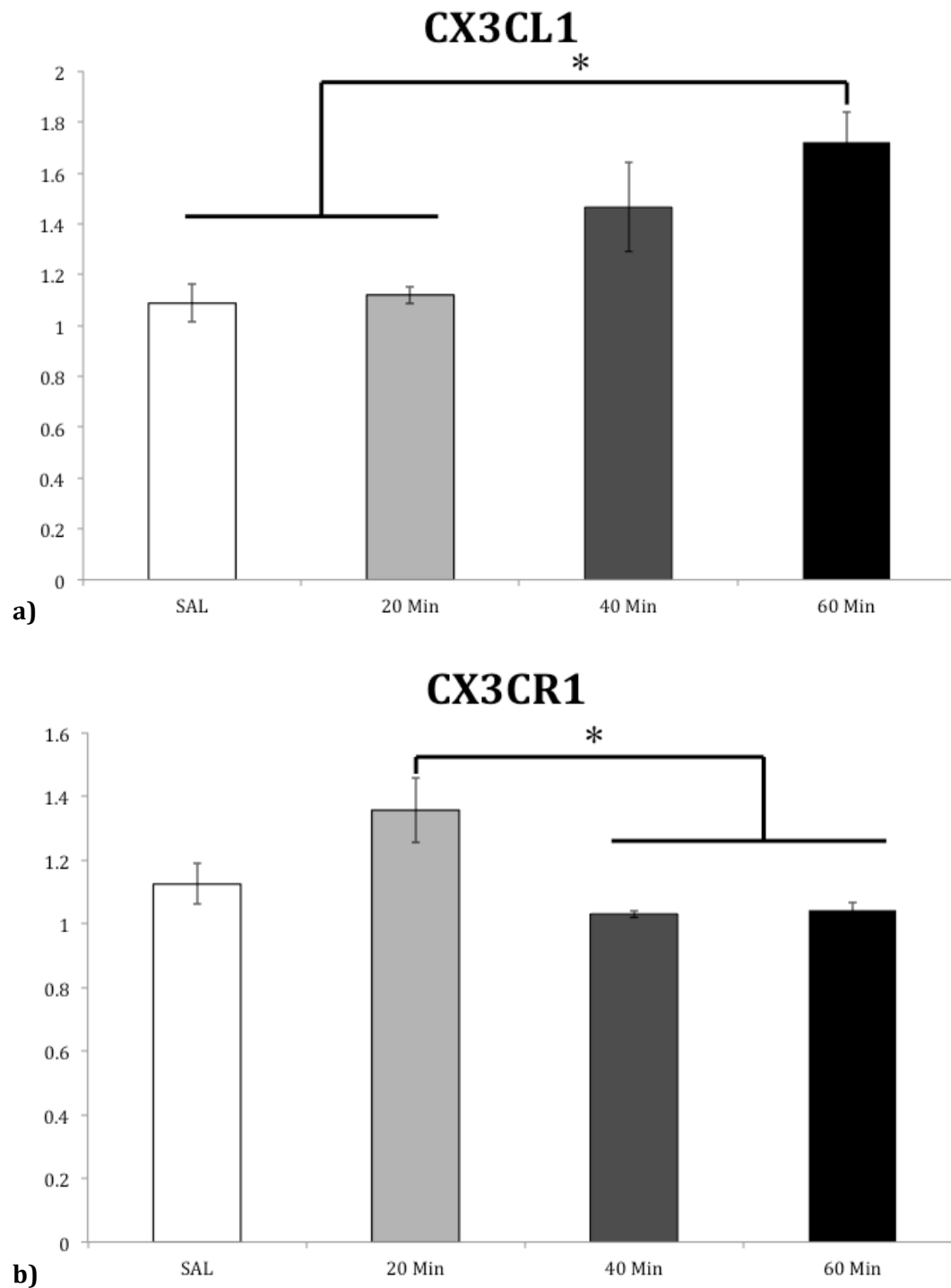


Figure 7. Increases in neuronal fractalkine expression in the NAcc, in response to morphine administration, mimics the response seen in rats [2]. Neuronal fractalkine (a) and neuronal fractalkine receptor (b) expression in the NAcc.

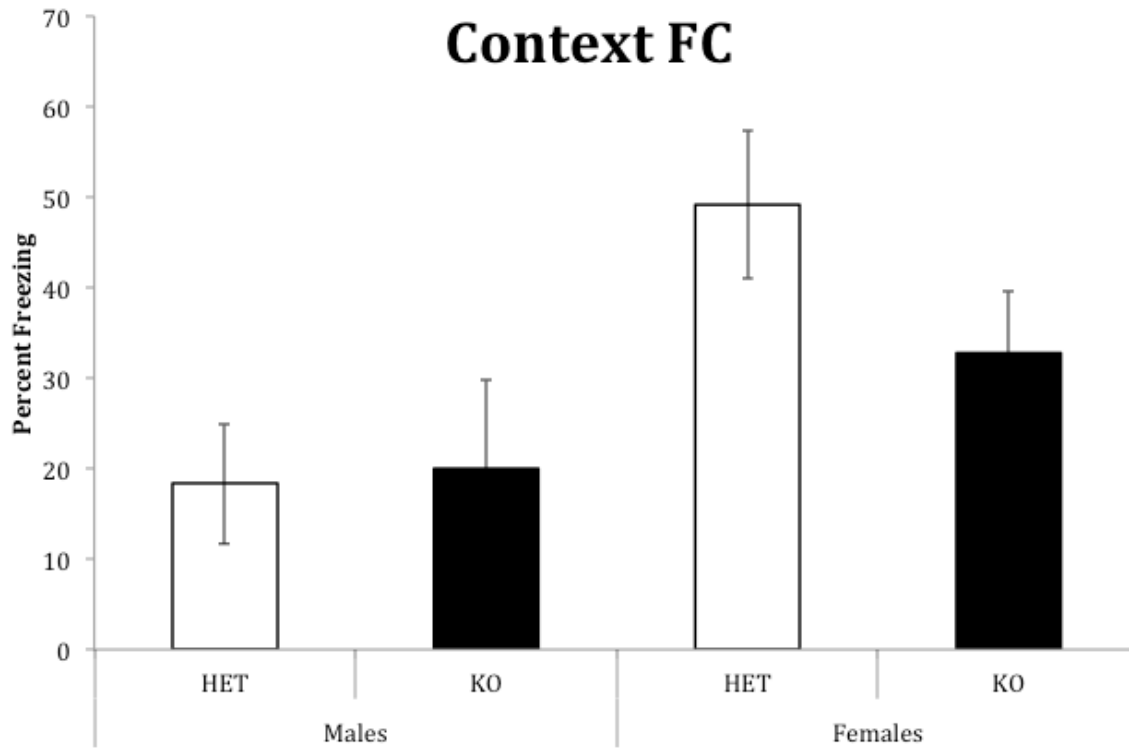


Figure 8. Contextual fear conditioning differs by sex, with females freezing significantly more than males ($F_{(1,19)} = 7.541$; $p = 0.014$). Percent freezing in TLR4 HET and KO mice is shown here in response to a contextual fear-conditioning paradigm.

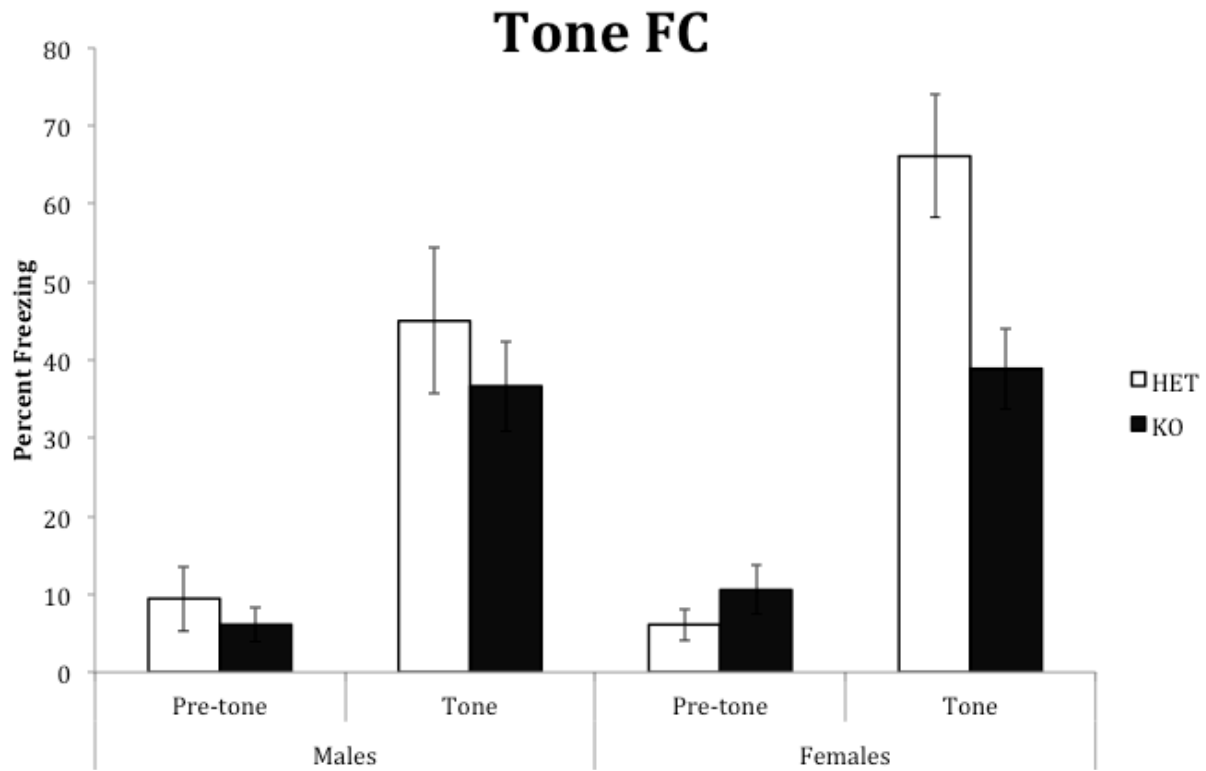


Figure 9. Percent freezing during conditioned stimulus conditioning (auditory-cued) shows a difference by genotype with KOs freezing significantly less than their HET counterparts ($F_{(1,19)} = 6.06$; $p = 0.026$). Percent freezing in TLR4 HET and KO mice is shown here in response to auditory fear conditioning paradigm.

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